CHROMATOGRAPHIC METHODS OF ANALYSIS—HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R. Raghavan
Jose C. Joseph
Abbott Laboratories, Abbott Park, Illinois, U.S.A.

INTRODUCTION

Chromatography is a technique to separate individual components in a mixture. High-performance liquid chromatographic (HPLC) methods are usually preferred over other methods of quantitative analysis. The methods are usually very specific to the analyte or analytes of interest since excellent separation of individual components are easily achieved. HPLC instruments are ubiquitous since the technique finds application in biotechnological, biomedical, clinical, and pharmaceutical analyses. Additionally, HPLC is used in many other fields including chemical, cosmetics, energy, environmental, and food industries. The availability of moderately priced, reliable, efficient, and sophisticated instrumentation has resulted in the use of HPLC as a method of choice in the pharmaceutical analysis, starting from the synthesis or isolation of a potential drug to the final stage of maintaining quality control information on a formulated dosage form.

Since the fundamental theoretical principles of HPLC were established in the 1960s, the development of HPLC instrumentation has been phenomenal. Development in column packing materials led to the development of reverse-phase chromatography in the 1970s. Development of computers and automation in the 1980s led to the ease of use of HPLC. In the 1990s the development of microcolumns, specialized columns, stable detectors, coupled with integrated data acquisition, storage, and retrieval capabilities has vastly increased the speed and efficiency of the HPLC instruments.

BASIC CONCEPTS, DEFINITIONS, AND CHROMATOGRAPHIC THEORY

Basic Concepts and Definitions

In HPLC for separation of individual components, the sample is introduced into a flowing stream of a liquid (*mobile phase*) and the analytes are allowed to pass through a layer column of packing materials of very small diameters (large surface area), called the *stationary phase*. As the analyte molecules pass through the column, carried by the moving mobile phase, there is constant interaction of the analyte molecules (or solutes) with the stationary phases as well as with the moving mobile phase. This results in a dynamic equilibrium. The differences in the equilibrium processes of the different solute molecules result in the separation of components of the mixture. When such separation is achieved by maintaining a constant composition of all the constituents of the mobile phase, the process is known as *isocratic elution*. If the mobile phase composition is changed continuously with respect to one or more of the solvents in the mobile phase, as a function of time, it is called *gradient elution*. When the *effluent* with mobile phase zones containing the analyte molecules emerges out of the column, it is passed through a *detector*, or a series of detectors. The detector signals respond as function of the solute concentration in the mobile phase zones. These signals are fed into *data processors*, which plot signal responses as a function of time. The graphic display of signals is called a chromatogram and the individual component zones are identified as *chromatographic peaks*. These peaks are characterized by the following parameters: their *peak widths, peak areas or peak heights,* and the extent of *tailing and the retention time of the peaks*. The instrumental set up is called a chromatograph. A typical chromatogram is shown in Fig. 1.

In HPLC solute molecules are introduced into a moving mobile phase stream. The stream passes through an inlet and emerges through the outlet of the column. Since the particles are extremely small in size (10 μm or less) and the column is fully packed, the moving mobile phase has to be pumped through using high pressure pumps. The solute molecules are only carried by the moving mobile phase. Molecules that interact with the surface of the column will be impeded and the emerging band will elute later than a band of weakly interacting molecules.

The relative migration of the solute is dependent on the thermodynamic and kinetic properties of the solute. The

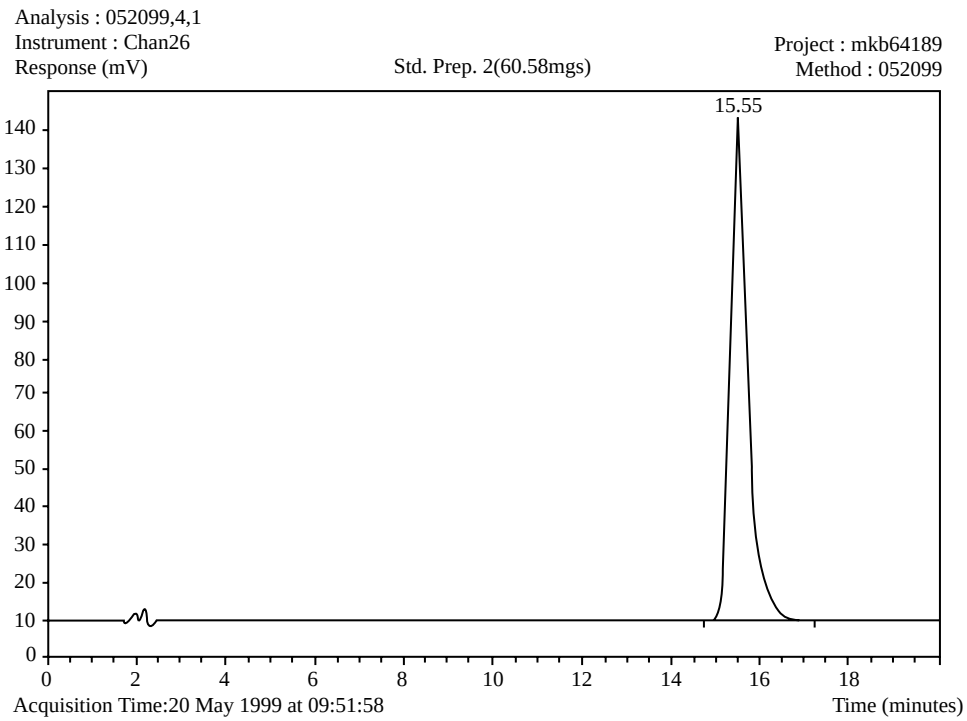

Fig. 1 Typical chromatogram.

extent and quality of separation of two closely eluting peaks are expressed by *retention* (*or capacity*) factor (k'), *selectivity factor* (α), and *the number of theoretical plats* (N). Capacity factor is a measure of time that the solute molecules are attached to the column particles, in comparison to that of the mobile phase. Thus greater the value of k', the greater is the interaction with the column particles. The capacity factor is dependent on the nature of the column, the organic or aqueous strength of the mobile phase, and the temperature at which the column is maintained. Experimentally measurable parameter of *relative retention time* with respect to the retention time of an active drug is computed. Under isocratic elution conditions, a value of 2–6 for capacity factor is optimal and normally values between 1 and 10 are acceptable. Greater the value of k', greater is the resolution between adjoining bands. However, as k' increases, there is increase in analysis time, which also results in lower detection limit, because of peak broadening. By using gradient elution these two disadvantages of isocratic elution can be overcome.

The chromatographic separation process is considered *efficient* if all the components are completely separated and the peak width is relatively narrow. Theoretically, when identical molecules enter the column head in a narrow band, the band width should be the same at the outlet. However, since solute molecules can elute at slightly different times because not all molecules will take the same path. These differences are caused by differences in the local surface area, relative physical activity of the interacting surface, the presence of stagnant mobile phase pools in crevices and pores, and minor variations of flow rate of mobile phases through these surfaces. Not all solute molecules traverse the same path and hence they might contribute to peak broadening.

Additionally, the quality of separation is evaluated by measurement of resolution, "R," between two closely eluting peaks.

$$R = 2(t_2 - t_1)/(w_1 + w_2)$$

where W_1 and W_2 are peak widths expressed in the same units as retention times, t_1 and t_2.

The greater the value of R, greater is the separation. For an R value of 1.00, solute purity is about 97.7% if each peak is Gaussian. In practice to attain a peak purity of 99.8% or greater a resolution of 1.50 is required.

The efficiency of separation, expressed as *theoretical plate number*, N, is calculated as follows:

$$N = 16(t_r\ w_B)^2 = 5.54(t_r/w_{1/2})$$

where "t_r" is the retention time, w_B, is the peak width at the base, and "w" is the band width at the peak height. A

column independent parameter, H (height equivalent to Theoretical Plate, $(HETP) = L/N$, where L is the length of the column), is more often used.

Column efficiency is inversely proportional to the particle size of the column packing. Thus the efficiency of separation will follow the following order.

$$E_{3\mu} > E_{5\mu} > E_{10\mu}$$

where E is efficiency and the subscripts denote particle size.

HPLC techniques can be used for preparative chemical separations. However, this discussion will be restricted to quantitative analytical separations. For quantitative analysis a known volume of a standard solution of known concentration is injected multiple times (most compendial methods require typically five to six injections). The average peak area of the peak of interest is computed. From a comparison of the peak area of similarly injected and separated analyte with that of the standard, the concentration of the unknown in the analyte is calculated. This procedure is known as *external calibration*. However, sometimes a known compound is added to both the standard and the analyte sample. Then the ratio of the relative peak area (or some times peak height) responses of the peak of interest, and that of the added compound are evaluated. From a comparison of the relative responses of the standard and that of the analyte injections, the concentration of the unknown is computed. This is known as *internal calibration*. Sometimes, peak height is used instead of peak area. The theoretical plates, resolution of two closely eluting peaks, percent relative standard deviation values of multiple injections, and tailing factor (extent of deviation of the chromatographic peak shape from symmetrical Guassian peak) are used as *system suitability parameters*. USP 24 (see bibliography), and other monographs provide examples of system suitability requirements and methods of measurement to meet the corresponding requirements.

COLUMNS AND MODES OF CHROMATOGRAPHY

The stationary phase in HPLC is the solid support contained in within a specified column over which the mobile phase flows effecting the separation of the individual components. The HPLC column is normally fabricated using 100- to 300-mm long stainless steel tubes with an internal diameter of 2–5 mm. They are packed with porous, microporous, spherical, or irregularly shaped particles, or particles with specific coatings with the following characteristics.

Mean particle sizes of 3–10 μm, surface area between 150 and 400 m^2/g, specific pore volume between 0.2 and 1.5 cm^3 or mL/g, and an apparent density of 0.4–0.6 g/ml. The different modes of chromatography are distinguished based on the differences in the packing materials, coupled with the corresponding compatible mobile phase components and the differences in the nature of the interacting functional groups present in solute molecules. These functional groups selectively and specifically interact with the column support material or mobile phase leading to selectivity and specificity of separation. The stationary phase chemical characteristics are altered by using suitably modified silica particles such that the differences in the functional group properties can be selectively utilized. These are summarized in Fig. 2. A brief description of the different modes of chromatography follows.

Normal Phase Chromatography

In normal phase chromatography, a polar stationary phase and a nonpolar mobile phase is used for separation. A modulator, like methanol or acetonitrile, at a suitable concentration can be used to increase the polarity of the mobile phase. Most normal phase chromatographic columns use bare silica support, which is acidic and polar. The acidic surface silanol groups, which are hydrophilic, interact differently with different functional groups in the solute molecule. The lipophilicity of the mobile phase also affect the preferential solubility or preferential adsorption on the surfaces. The use of silica support in normal phase chromatography suffers from the following disadvantages: 1) product dependent activity of silica leading to poor separation and variations from column to column and from brand to brand; 2) irreversible adsorption of strong polar solutes on the column support; 3) the necessity to control the water content of the mobile phase; and 4) slow re-equilibration of particles to mobile phase changes. Some of these problems are over come with the use of modified silica stationary phases. The silica surfaces are modified by bonding with appropriate functional groups. Cyanoalkyl or aminoalkyl or phenyl moieties are bonded to these surfaces.

Non-polar supports like polystyrene/divinylbenzene copolymers or carbon are also used as column materials. Alumina is polar and acidic while TiO_2, and zirconia are much more neutral. They all have good aqueous stability compared to silica. Normal phase chromatography is restricted to the separation of stereochemical isomers, diastereomers, low molecular weight aromatic compounds and functionalized long chain aliphatic compounds.

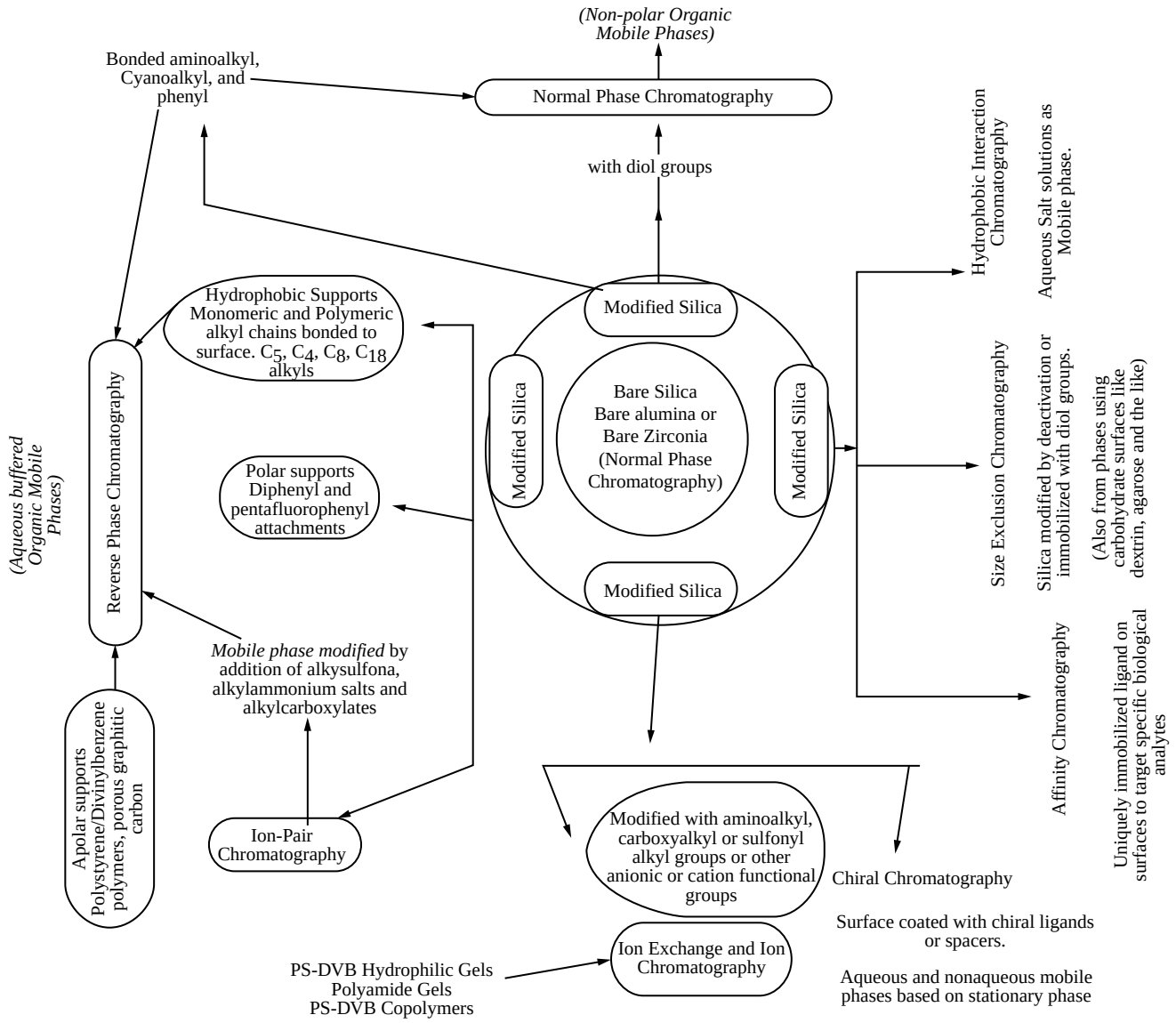

Fig. 2 Silica surface and different modes of chromatography.

Reverse Phase Chromatography

Since many compounds of pharmaceutical interest are generally polar and highly water soluble, reverse phase chromatography is extensively used. In reverse phase chromatography, separation is accomplished by the use of polar mobile phases on nonpolar stationary phase. By chemically bonding silanol groups in silica, nonpolar stationary phases are obtained. Typically C_3, C_4, C_8, C_{18} alkyl chains are bonded to silica support surfaces. Mobile phases are usually buffered aqueous solutions containing one or more of the organic solvents like methanol, or acetonitrile, or tetrahydrofuran as modulators. The modulators reduce the polarity and decrease retention of solutes. Depending on the organic solvent used selectivity also can be modified. Column designations like octyl (C_8), octadecyl (C_{18}), refers to the length of the carbon chain attached to the silica surface. Amino, cyano, and phenyl columns can also be used in reverse phase chromatography. To increase polarity of the stationary phases diphenyl and pentafluorophenyl columns are also used. Alkylated polystyrene/divinyl benzene polymers also can be used instead of silica based supports.

In reverse phase chromatography separation may be due to either adsorption effects or due to partitioning of the solute between the stationary phase and the mobile phase.

More often, separation is probably based on both mechanistic pathways; the relative contribution of each for a specific separation process cannot be estimated. In general, C_{18}-bonded phases yield better retention and better separation compared to C_8-bonded phases because of higher carbon content in the stationary and higher non-polar interaction with the solute.

In isocratic elution, some times compounds are not fully resolved and some compounds are highly retained. The sensitivity of the highly retained compound is reduced considerably due to peak broadening. These drawbacks are overcome when gradient elution is adopted. In gradient elution, the sample is injected when mobile phase has low organic content. The organic fraction is then increased in increments to decrease polarity. These increments are usually linear. Multiple step gradients are also adopted. The capacity factor is dependent on flow rate, slope of the gradient and column dead volume. In linear gradient elution, the bandwidth is generally constant and hence the peak becomes sharper, yielding enhanced sensitivity.

Reverse phase liquid chromatography is very versatile, fast and highly reproducible. Aqueous solutions are normally used and the modifiers used are very cheap and highly pure. Separation is predictable based on the polarity, pH profile, solubility and other physicochemical characteristics of the solute molecules. Analysis time is rather short and reequilibration is generally fast. Multiple components with minor differences in polarity can be separated by appropriate choice of gradient profiles.

Stationary phases have been modified to

1. Reduce interaction of free silanols
2. Improve the stability of phases over a wide range of pH
3. Introduce functional groups on the phases that will enable prediction of selectivity for different solutes.

Only about 50% of the free silanols in silica are bonded in octyl and octadecyl stationary phases. These residual silanols contents is further reduced by a process called *endcapping*, in which hexamethyl and isobutyl groups are additionally introduced into the matrix. Endcapped columns offer better separation and retention in addition to reduced peak tailing.

Although endcapped columns offer some advantages, the aqueous instability is still problem. To over come these problems of silica based columns, alternative supports like alumina (Al_2O_3), zirconica (ZrO_2), and titania (TiO_2) have been developed. Alumina columns are stable in the pH range of 2–12, while zirconia columns extend the range from 0 to 14. These are basic oxides and hence silanol-like interactions are eliminated. Different bonded phases can be obtained using zirconia, however, because of poor reactivity such bonded phases are difficult to prepare with alumina supports.

Polydivinylbenzene and polystyrene polymer based stationary phases also eliminate these effects. Porous graphitic carbon provides a highly nonpolar surface with excellent chemical stability under acidic and basic conditions, However, they suffer from lower sample loading capacity and lower efficiency than conventional columns.

In spite of these efforts, silica based modified columns are still widely used. Many column-manufacturers have introduced quality control procedures for the synthesis and characterization of silica based columns. Therefore, lot to lot variations from the same manufacturers have been considerably reduced. However, identical phases from different manufacturers can yield different separation behaviors of the same analyte under identical instrumental and mobile phase conditions. (Of 13 phenyl columns investigated for an oncolytics, we found only three columns providing similar separation profile for a 13-component impurity mixture.)

Ion-Pair Chromatography

In reverse phase chromatographic separations, ionic compounds, being more water soluble, are not retained in the column. To increase retention and separation a strong counter ion (an organic alkyl or aryl-substituted ion of opposite charge) is added to the mobile phase. Typically alkane sulfonic acid salts or alkyl ammonium salts are added to the mobile phase. These counter ions associate (ion-pairs) with the analyte ion, while displacing the inorganic counter ion like chloride ions. Analyte is retained since the ion-pair partitions into the stationary phase like a large non-polar neutral organic molecule. This technique, also known as *ion-interaction chromatography*, utilizes the effect of pH, ionic strength, mobile phase organic content and temperature to control retention and separation.

Ion-Exchange and Ion Chromatography

Ion-exchange stationary phases consist of solid resin particles that have positive or negative ionic bonding sites incorporated in the stationary phase. The ions of opposite charge in the mobile phase are exchanged with ions on the surface. The ions of opposite charge in the mobile phase are exchanged with ions on the surface. Cation exchange resins contain covalently bound negatively charged functional groups, while anion exchange resins have positively charged functional groups. When the charged

functional groups is a sulfonate anion, it is called strong cation exchanger. Week cation exchange resins contain such functional groups as carboxymethyl, phosphate, slulfoalkyl groups. If strongly basic quaternary amines are on the resin, it is called a strong anion exchanger. Weak anion exchangers contain weakly basic groups like aminonethyl, diethyaminomethyl groups. If these functionalities are only on the surface of the stationary phase they are called pellicular particles. When pellicular particles are used, lower eluent concentrations are adequate. When pellicular ion-exchange resins are used, ion-exchange is the only method of separation. When ions are thus separated, particularly in the separation of inorganic ions or small organic acid anions, it is called *ion chromatography*. Most modern ion chromatographic stationary phases use polystyrene divinylbenzene copolymer resins. These stationary phases have very high pH stability and can withstand strong acids and bases.

Using ion chromatographic separation and conductivity detection the inorganic anions like halides, phosphate, nitrite, nitrate, thiocyanate, and sulfate and many cation ions including transition metal ions can be detected. When quantitation of ions are carried out in a solution matrix that is weakly conducting, coductomertric detection and quantitation is possible since the total background conductivity is very small. Examples include the determination of ions in sea water or tap water or from environmental streams. However, if strongly acidic or basic eluents are used, the background conductivity is high. In order to suppress the background conductivity, special suppressor columns are used, which neutralize the acids or bases after elution and before detection. New pulsed amperometric detectors (PAD) are commercially available. With the use of PADs, parts per billion levels of metal ions can be detected. Accurate quantitation of metal ions is possible since ready to use inorganic calibration standards are commercially available.

When metal ions are used as counter ions, instead of organic quaternary ammonium ions, in the packing material, the hydroxy (–OH–) functional groups of the carbohydrates and other sugars interact with these metals ion. Pb^{2+}, Ca^{2+}, and Na^{+}, are typical metal ions in the packing material. Depending upon the type of counter ions used, the intensity of the interaction changes and therefore, the retention between different carbohydrates vary. Some of the carbohydrates are also retained because of the size of the molecule under these conditions.

Ion-exchange chromatography is widely used for analyses of proteins, glycoproteins, peptides and other high molecular weight compounds. These organic compounds have considerable surface charge and behave like charged anions. Hence they are amenable to ion exchange separation. To separate nucleotides of similar molecular structures, the differences in the phosphate groups of various nucleotides and the differences in their binding characteristics are used. In addition to silica based resins, acrylic polymer based resins, dextrans, and cellulose bonded phases are used for the separation of proteins. In order to preserve the biological activity during separation, hand poured columns packed with ion-exchange materials are used. Gravity flow of eluent at low temperatures is the norm for separation. Thus, ion exchange chromatography and ion chromatography are no more used synonymously.

Hydrophobic Interaction Chromatography

In hydrophobic interaction chromatography, weakly hydrophobic sorbents are used. Gradient elution with decreasing concentration of salt is used for the separation of large biomolecules, particularly proteins, by this technique. The non-polar functional groups of large biopolymer molecules (weakly) associate with the hydrophobic ligands in the stationary phase. The stationary phase consists of a highly hydrophobic organic layer. The organic layers contain short alkyl or aryl functional groups attached at the surface. These attached groups are separated with large unattached space in between these attached functional groups. Because of this wider spacing, these "*soft*" stationary phases preserve biological activity without denaturing the proteins. High ionic strength aqueous mobile phases enhance binding between the solute and the stationary phase. Then the salt concentration is decreased to decrease the ionic strength of the mobile phase. The weak mobile phases then reduces the binding and thus separation is effected.

Typical stationary phases include the following: polyvinylpyrrolidone (PVP) coated silica sorbents, monodisperse nonporous silica columns with surface bound amides or ethers and composite agarose and polyacrylamide gels. The eluent normally consists of salts at concentrations greater than 1.0 M. Typical salts include sodium phosphate, sodium sulfate and ammonium sulfate, and organic acid salts like monosodium glutamate. Protein retention is stronger with salts that increase surface tension like phosphates, sulfates, citrates, which are solvated in water than with salts such as perchlorates and thiocyanates and the like.

Typical biological compounds that are separated by HIC include, cytochrome P-450, enzymes, DNA polymerase, epidermal growth factor, glycoprotein hormones, human immunoglobulins, human recombinant DNA and canine pancreatic juice proteins. Many HIC techniques have been used for large scale purification of proteins.

Affinity Chromatography

This chromatographic technique uses a specific binding agent. The stationary phase is prepared by immobilizing one of a pair of interacting molecules on to particles of support. The immobilized molecule is referred to as a *ligand*. These ligands selectively bind to the interacting second pair in a protein or a biomolecule. For example, an antitransferrin antibody is immobilized on the support. In this example, the antibody is the ligand; the transferrin antigen in the biomolecule will bind to the surface or release out of the surface depending on the mobile phase strength. Therefore, this technique, which utilizes the differences in the affinity of the two specific interacting groups or moieties is called *affinity chromatography*.

Typical ligands may be of biological origin like antibodies, inhibitors, substrates, coenzymes, cofactors, nucleic acids, and the like, or of nonbiological origin like triazine dyes, metal chelates, boronate salts, etc. In this technique, sample is injected on to the column using a weak mobile phase called the application buffer. Under these conditions, the only interacting component is bound to the surface and hence retained in the column. The rest are washed out of the column. Then using a stronger mobile phase, called the eluent buffer, the solute of interest is released, eluted from the column, and then quantitated or collected for later use. Elution may involve two separate steps or may be a simple step gradient. This technique is used for the separation of hormones, peptides, proteins, viruses, enzymes, glycopeptides, antibodies, metal binding amino acids, etc. Afinity chromatography is further classified as bioaffinity, bioadsorption, immunoaffinity and the like depending on the nature of the ligand on the support.

Size Exclusion Chromatography

Size exclusion chromatographic (SEC) technique is used for the separation of biomelcules based on their molecular size. Synthetic and many natural polymers like polysaccharides, cellulosics, natural rubber, and some proteins have chains of differing molecular weight components. When such mixed molecular weight species are present it is said to be a *polydisperse* polymer. Otherwise, the monomer is said to be monophasic. The SEC chromatographic peak is broad indicative of the elution of the different components of the polydisperse phase. The polydisperse phase is described by up to "3" molecular weight parameters that define the distribution of species. These are 1) number average, *Mn*; 2) the weight average, *Mw*; and 3) z-average, *Mz*, molecular weights. When $Mn = Mw$, the distribution is said to "Monodisperse." *Mw*/*Mn* is a measure of the polydispersity of the system. For large biomolecules, *Mn* and *Mw* are different since *M*w is usually higher, because it is sensitive to the presence of high molecular components in the distribution. *Mn*, *Mw*, *Mz* are defined as follows:

$$M_n = \Sigma N_i M_i / \Sigma W_1$$

$$M_w = \Sigma M_i W_i / \Sigma W_i$$

$$\text{and } M_z = \Sigma W_i M_i^2 / \Sigma W_i M_i$$

where *Ni*, is the number of molecules of molecular weight M*i*, and W*i* refers to the weight (or concentration) of M*i*. The ratio of *M*w/*Mn* or *Mz*/*M*w shows the width of the distribution. Size exclusion chromatography is a relative and not an absolute technique.

Gel permeation chromatography (GPC) refers to the technique in which polymers that are soluble in organic solvents are separated. In These cases, more polar organic mobile phases like tetrahydrofuran, toluene, chloroform will be used. Gel filtration chromatography (GFC) is used for separation of water soluble biopolymers. Four different calibration methods are used. If absolute known molecular weight standards are used, it is called *primary calibration method*. In *secondary calibration approach*, poly-dispersity standards of material similar to samples are used. The result is then usually specified as apparent molecular weight distribution. When *M*w and *Mn* are obtained by use of an iteration procedure using a sophisticated software program, it is called *broad molecular weight calibration*. The iteration procedure uses calibration slopes and intercepts of broad molecular weight standards with known *Mn* and *Mw* values. *Universal calibration* is obtained from a plot of log ($M\eta$) vs. *V*e, elution volume, where η, is the intrinsic viscosity of the polymer measured at the same temperature and in the same solvent as used for the mobile phase. This technique uses on line SEC viscometers in conjunction with universal calibration.

For organosoluble polymers cross linked polystyrene or silica based packings are used. For water soluble polymers various silica based and hydrophobic polymeric packings are used. Pore sizes of the SEC packings may range from 3 to 300 nm.

In addition to refractive index detectors, specialized detectors such as on-line SEC detectors and low angle laser light scattering detectors are used for determining the distribution of molecular weights by SEC.

INSTRUMENTATION

Solvent(s) Delivery

Solvent (mobile phase) delivery is achieved using high pressure pumps. There are several types of pumps

commercially available for delivery of mobile phase through the injector, column, detectors and then to the solvent waste container. Since the column head pressure is high, the pumps operate under high pressures (50–300 psi). Most commonly used pumps are reciprocating piston pumps with check valves. These pumps are usually computer controlled. The modern pumps provide flow rate precision which is better than 0.1% in retention time.

For gradient elution at least two solvents has to be pumped and then mixed before it passes through the column. Additionally, the solvent composition is changed in a continuous linear, continuous nonlinear, or stepwise fashion. The solvents are mixed with the use of proportioning valves, and the mixed solvents reach the pump. Since solvent mixing occurs before the pump under low pressure conditions, it is called low pressure mixing. This is the most commonly used form of mixing for gradient elution. In high pressure mixing, two or more solvents are individually pumped using different pumps and then they are mixed at high pressures. Pump head leakage is a common problem in such pumps. The pump heads has to be constantly monitored or repaired for leakage.

Dissolved oxygen from mobile phase solvents has to be removed. This is accomplished by passing helium gas through the solvent container or by passing the mobile phases through helium purging units. Currently, computers that control the pumps also control vacuum degassers, which are placed in between the pump and solvent reservoir.

Autosamplers

Autosamplers are used for unattended introduction of samples from vials that are arranged either in a rectangular or circular tray. Autosampler delivers the desired volume of 1–2.5 ml with a precision of less than or equal to 0.5% for injections greater than 10 μl. All autosamplers use mechanized valves. Majority of HPLC autosamplers belong to one of two types. In type I autosamplers the septum cap is pierced using a syringe needle, liquid is displaced into the syringe by gas pressure or plunger action to the inlet port of a six port valve injector. The filled syringe is withdrawn, moved, and the solution deposited into flowing stream of mobile phase by the use of appropriate electromechanical devices.

Type 2 autosamplers behave in a very similar way to type I except partial loop volume can be filled and delivered. Additionally, this allows the injection of low volume with high precision compared to type I autosamplers. Both types of autosamplers suffer from carry over problems if appropriate wash cycle and wash solvents are not used between each sampling from the vials.

Detectors

A liquid chromatography detector consists of sensors and an associated electronic device to send signals to a processor. Detectors are classified as bulk property detectors or solute property detectors. Bulk property detectors measure the changes in the property of the combined eluting mobile phase and the eluting solute. For example, the refractive index is characteristic of a liquid. When a solute is dissolved, the refractive index of the solution is different from that of the solvent. These change the property of the bulk solution. Although the change is due to the presence of the solute, the refractive index of the bulk as a whole is different from that of the pure solvent. Refractive index detectors and conductivity detectors are examples of bulk property detectors. Solute property detectors detect the changes in some physical or chemical property of eluting solute component of the mobile phase.

HLPC detector

A HPLC detector should have the following characteristics.

1. Excellent linear response as a function of concentration of the solute.
2. Wide linear dynamic range; the dynamic range over which the response to concentration is linear.
3. High signal to noise ratio. The noise arises as a result of fluctuations or perturbations caused to the signal as a result of temperature, pressure, or flow rate changes in the mobile phase. Noise is also caused by the electronic circuits used in the detector system. All these combined perturbations, called noise, should be low such that very low concentrations of solute can be detected.

Refractive index detectors

The most common Refractive Index (RI) detector uses a differential refractometer, which responds to the deflection of a light beam; the deflection being caused by the differences in the refractive indices of a cell through which eluant passes and that of a reference cell in which the mobile phase is contained. The response of the detector is proportional to the mass concentration irrespective of the nature of solute being analyzed.

Conductivity detector

The conductivity detector measures the conductivity of a solution containing an electrolyte. When current is allowed to pass through two electrodes, there is resistance (or better impedance) to the flow of current through the medium. This impedance decreases if conducting electroytes are present in the eluant. This detector is mostly used in ionchromatography. Coupled with ion

suppression technology, this has become a versatile detector for low levels of inorganic ion content in analytes of interest.

UV–vis detectors

UV–vis spectophotometric detectors are most commonly used detectors in HPLC, since most organic compounds absorb light in the UV region (190–400 nm) and a few in the visible region (400–750 nm). Fixed wavelength, variable wavelength, and diode array detectors are commercially available. All these operate based on the ability of a solute to absorb light at defined wavelengths based on the chemical structure and functional groups present in the solute molecule. The source of UV light is a deuterium or high pressure xenon lamp while for the visible range it is a simple tungsten lamp.

A beam of light is allowed to pass through a flow cell mounted at the end of the column. As the solute molecules elute from the column and enter the flow cell, they absorb radiation. The differences in the light energy, as a result of absorption, are used as a measure of quantitation. Fixed wavelength detectors operate at a single wavelength, either at 254 or at 280 nm in the UV region. In variable wavelength detector using a monochromator light of a particular wavelength (less than ± 3 nm) can be selected, passed through the sample, and then on to a photocell for detection. Currently available detectors can be programmed to change wavelengths while analysis is in progress to get a spectrum of the eluting species. Otherwise, using appropriate software, the absorbence of the eluate can be monitored simultaneously at two to four different wavelengths. This multi-wavelength detector is less sensitive compared to fixed wavelength detectors (10^{-7} g/ml vs. 5×10^{-8} g/ml).

Photodiode array detectors

Diode array detectors acquire data over the entire range of UV–vis range 190–800 nm; in some up to 1100 nm. Two different types of photodiode array detectors are available in the market. In one, to detect over an entire spectrum, light from a continuous source is passed through the cell using a rapidly rotating or vibrating grating, which passes radiation through the cell, one wavelength at a time. The signal of the photodetector is measured as a function of time over the measuring cycle. Then, from the measuring cycle, wavelength is related to time to obtain a plot of wavelength vs. signal. In the second type polychromatic light is passed through the cell and then through a holographic grating. The light dispersed from the grating is arranged to fall on a linear photodiode array. These diode array detectors are very versatile and are used to:

1. Check peak purity using "peak overylay" (normalized) methods or by computing peak ratios at two different wavelengths
2. Identify peaks by spectral matching with accumulated and stored spectral libraries
3. Generate the spectrum of the eluting peak and determine wavelength of maximum absorption of an unknown or impurity peak
4. Quantify different peaks at different wavelengths in a single chromatographic run
5. Provide graphic 3D or contour plot presentations to regulatory agencies to show the purity of the eluting chromatographic peak
6. Identify peaks, during method optimization, when the order of elution of the compounds changes.

It should be noted that the sensitivity of these detectors is lower than fixed or variable wavelength detectors. Also, if an overlapping impurity is present either in the fronting or tailing portions of the eluting peak, it can be detected only when the concentration of the impurity is greater than 2.0% relative to that of the major peak.

Fluorescence detectors

Since the fluorescence detector is a highly sensitive, picogram levels of solute can be detected by using this detector. However, this is limited to compounds that naturally fluoresce or can be made to fluoresce by reacting with suitable derivatizing agents. Even this is restricted since appropriate functional groups that undergo such derivatization should be present in the solute molecule. In many HPLC methods where this technique is utilized, the fluorescent agent is added after separation of the components. This has the advantage that the derivatization need not be quantitative. But the reaction should be very rapid, reproducible and proportional to concentration. Because it is an unique property of the solute, it offers selectivity as well as specificity of detection.

Electrochemical detector

This is also a very specific and extremely sensitive detector. The specificity arises from the need to have an electro-oxidizable or reducible functional group present in the solute molecule. Similar to the case of fluorescence detectors, solute molecules can be derivatized to yield compounds containing oxidizable (or rarely, reducible) functional groups. A desired potential is applied between a working electrode and a reference electrode connected to the flow cell. A third electrode known as auxiliary electrode is used to control the potential. As the oxidation takes place at the working electrode surface, the current

flow changes. This is monitored, amplified and presented as response using appropriate software and hardware. When the oxidation reaction is allowed to go to completion using a high surface area of working electrode and exhausting all the reactant in the flow cell, it is called a *coulometric* detector. In this case, the total number of coulombs of charge transferred is measured. However, in the most common *amperometric* detector, the solute molecules at the surface and those are very close to the surface are oxidized by maintaining the working electrode at a constant potential. This oxidation process is diffusion controlled and is proportional to concentration. Here the increase in the current flow, "*i*" is measured, amplified and the signal is presented as a function of time. Glassy carbon electrode is the commonly used electrode for oxidation. Surface coating and the resulting contamination of the surfaces leads to a decrease in sensitivity on constant use. Therefore, this detector has be disassembled, and cleaned very often. Also this requires long equilibration time compared to other detectors. Therefore, it is not an extensively used detector compared to other detectors described in this article. The problem of electrode pollution is overcome in pulsed amperometric detector (PAD). In this technique using a gold or platinum electrode a repeating cycle of potential pulses are applied. Typically, in a one second pulse cycle, three potential pulses are applied. In the initial negative pulse the solute is adsorbed; in the second positive potential pulse the adsorbed compounds are oxidized and the increased current as a result of oxidation is measured. In the third pulse at a high much higher positive, the electrode surface is cleaned by oxidation of the electrode itself. Thus the new surface generated is used for the repeat cycling process. This is very useful in the detection of very low levels of sugars and other polyhydroxy compounds that are otherwise are not easily oxidized.

Evaporative light scattering detector

In this detector, the entire eluate from the column is atomized and evaporated to form small droplets. The solutes finally remaining form particulates suspended in the atomizing gas. When these particles are allowed to pass through a light beam, light is scattered in all directions by the particles. This is known as Rayleigh scattering. However, the light scattered at 45° angle to the incident beam is viewed using appropriate optical filters and the resultant signal is electronically processed. The detector response is sensitive to the mass of the solute particles and hence it is a universal detector. The sensitivity of this detector compares with that of the RI detector.

Computers

The computers are ubiquitous and have become indispensable component of any laboratory. It serves both data processing and process control functions. As a data processor, the computer receives, stores, archives and reprocess the input signals from various detectors. Additionally, as a system processor, the computer monitors the system detectors. The data acquired for each chromatographic run can be processed appropriately to arrive at peak area or peak height or response ratios to an added internal standard. From these it can be used to calculate the concentrations of individual components in an analyte.

Through the use of appropriate software and hardware, the computer can be programmed to do the following: 1) to command injection of the samples; 2) to control and monitor, various parameters of the pump like the flow rate, composition of the mobile phase, column pressure; 3) to monitor and control column oven, and detector temperatures; and 4) to start and stop injectors, detectors and other system units. The computer also can be used to monitor system suitability parameters and reinject samples after adjusting conditions to meet system suitability. A decision tree can be constructed to allow retesting when preestablished system suitability conditions are not met. The computer can be programmed such that if conditions are not met, the system is shut down or paused until it can be attended to. Sample preparation, derivatization and other processes also can be controlled using individual computers. With multiple computers attached to a large computer called the *server*, data from a number of detectors can be monitored, stored, and archived. The computer is also used as an excellent book-keeper storing all the information regarding samples, their results, and also the conditions under which those results are obtained. The computer has become versatile tool in the highly regulated pharmaceutical industry especially to provide traceability and data integrity to required government and compendial agencies such as FDA, USP, BP, etc.

METHOD DEVELOPMENT AND METHOD VALIDATION

The method development process involves selecting appropriate method conditions for the sample in hand. It is based on prior knowledge of the sample properties, pK_a or pK_b values of functional groups, the polarity and size of solute molecules, UV–vis spectral properties, redox behavior, concentration range, solubility behavior and the like. From a knowledge of these, suitable mode of chromatography, corresponding column(s), mobile phase

composition, flow rate, choice of detectors, gradient or isocratic conditions, and the like can be selected. Once the method has been developed with some initial trials, optimization is carried out. Optimization is necessary to accomplish best possible separation of all components within the shortest possible time or in the case of low level detection, conditions have to be optimized such that required level of detection and/or quantitation can be achieved. In general, the system suitability parameters are usually evaluated and specified before method validation is performed.

Method validation is a process by which documented evidence is prepared and provided to show that the method meets the intended need. Highly regulated pharmaceutical analytical laboratories perform method validation and generate data on the following parameters, to comply with the compliance requirements of government agencies such FDA, EPA and/or to provide data for compendial agencies like USP, BP, etc.

The parameters that define validation are accuracy, precision, specificity, linearity, ruggedness, and robustness. Accuracy is a measure of the closeness of the measured value to the true value or an accepted reference value. This is usually measured by spiking known amounts of the analyte to a matrix called the placebo, and computing the recovery of the analyte after sample analysis. Placebo contains all the ingredients of a formulation other than the active ingredient or the ingredient being analyzed. FDA and ICH (International Committee on Harmonization of Technical Requirements for Registration of Pharmaceutical for Human use) guidelines recommend collecting data from nine determinations at (at least) three concentration levels encompassing the range of target analyte concentration.

Precision refers to the degree of repeatability under the stated conditions of the method. It is expressed as percent relative standard deviation (% RSD) for a statistically significant number of analyses of samples. Precision provides a measure of day to day, analyst to analyst and instrument to instrument variation on a routine basis. The precision data provided in support are standard deviation, % RSD, confidence intervals and may also include inter laboratory variations.

Specificity refers to the ability to determine the concentration of the analyte with a high degree of confidence that the other components in the matrix do not interfere with the target analyte. The potential interfering substances include other active and inactive ingredients, impurities, degradation products of the components and active ingredient, and extractables from the container-closure system and the like. Specificity is the currently accepted terminology by regulatory agencies. In the literature selectivity is also used to suggest specificity. [Refer ⟨1225⟩ of USP XXII (1900) vs. XXIII (1995) and 24 (2000).]

Linearity refers to the linear response of the detector to the analyte concentration within a specified *range*. Range, expressed in the same units as the analytical test results, is the interval between the lower and upper levels of the analyte concentration. To show linearity, a plot of response vs. concentration is provided for at least at five different concentration levels within the range. In addition, the slope and intercept of the regression line with the correlation coefficient are also provided. Normally a single standard is recommended if the intercept is very close to zero. Otherwise, quantitation, based on linear plot of multiple standards, is generally recommended.

Ruggedness is again a measure of precision. ICH guidelines include ruggedness in precision, while USP separates it. Ruggedness according to USP is a measure of the variation in interlaboratory comparison data. It is performed to establish lack of influence of test results based on operational and environmental parameters.

Robustness is a measure of how method parameters like organic content, pH, ionic strength of the mobile phase and column temperature do not affect test results when minor variations are deliberately induced in these parameters. The quality of separation of components, the accuracy and precision of the method and the like should not change as a result of these variations. (It is the belief of the authors that robustness should be part of the method development process regarding the appropriate choice of the column and the mobile phase conditions.)

Two other terms, limit of quantitation (LOQ) and limit of detection (LOD) are used especially in the determination of the analytes at trace levels. The trace level analyte may be impurities or degradation products of ingredients in a sample or it may be a solution of active ingredient at trace levels in rinse or swab samples obtained as part of cleaning validation. LOD refers to the lowest concentration of an analyte that can be detected (but not quantitated), under a given set of chromatographic conditions. Usually, it is expressed as a concentration parameter calculated using a signal to noise ratio of 3:1. LOQ refers to the concentration of the analyte that can be quantified in a sample with a predefined value of variation in precision. Normally accepted signal to noise ratio value for LOQ is 10:1. Generally, LOD and LOQ values are dictated by specific requirements, in order to ascertain reproducibility of LOD and LOQ values. Examples of specific requirements include determination of impurities at 0.05% using a 0.1% surrogate standard, or ppm quantitation or detection requirements based on equipment cleanability and the like. For practical purposes, it is

recommended that a control solution at a known concentration of the analyte be prepared such that a peak corresponding to this analyte concentration is always detected (LOD) or quantitated (LOQ). From one of these two parameters, if experimentally obtained, the other can be calculated.

PRACTICAL CONSIDERATIONS

Sample Preparation

For many analyses, sample preparation may simply involve dissolving a known weight or volume of sample and diluting to appropriate concentration for analysis. However, extensive sample preparation may be required, if precolumn derivatization is needed. Precolumn derivatization is carried out to increase sensitivity, to induce specificity, or to separate optical isomers and to reduce or eliminate otherwise complex and expensive clean up procedures. Solid phase extraction using appropriate solid cartridges are normally used in the clean up of biological samples. Solid phase cartridges can also be used to increase the concentration of trace analyte. These purification steps can be done in situ during analysis by appropriate plumbing using six- or ten-port valves and using computer control of the pumps and values. No matter how it is done, attention must be paid for proper sampling and preparation of samples.

Mobile Phase Preparation

Mobile phase should be prepared using HPLC grade solvents and analytical reagent chemicals only. Mobile phases should be filtered using ($\leq$0.5 μm) filters to remove any particulate matter from the solvent. To eliminate dissolved air, helium sparging or vacuum degassing is necessary. In gradient elution, in order not to alter solid phase wetting and gelling characteristics, it is advisable to use at least 5% water content instead of pure organic solvents as one of the mobile phases.

System Maintenance

Pumps and columns should be cleaned of with 50:50 methanol–water or acetonitrile–water mixture to eliminate buffers from the system. Pumps, injectors, and detectors should require periodic and scheduled maintenance to keep instruments in good operating conditions. Detector should be calibrated on a regular basis using appropriate calibration standards available from NIST or other sources traceable to NIST. FDA and other regulatory agencies require extensive documentation regarding the "health" of these instruments. Therefore, necessary and appropriate documentation should be maintained regarding details of maintenance. If there is any instrument failure additional documentation is necessary to prove that analytical results were not impacted or compromised because of these failures, if any.

BIBLIOGRAPHY

Barth, H.G.; Boesm, B.E.; Jackson, C. Anal. Chem. **1998**, *68*, 455–466.

Berthod, A. J.Chromatogr. **1991**, *549*, 1.

Bidlingmeyer, B.A. J. Chromatogr. Sci. **1997**, *35*, 392–400.

Brown, P.R., Grushka, E., Eds. *Advances in Chromatography;* Marcel Dekker, Inc.: New York, 1998; 39.

Hanson, M.; Unger, K.K. LC-GC **1997**, *15*, 364–366, 368.

Katz, E., Eksteen, R., Schoenmakers, P., Miller, N., Eds. Chromatographic Science Series. *Handbook of HPLC*; Marcel Dekker, Inc.: New York, 1998; 78.

Katz, E.D., Ed. *High Performance Liquid Chromatography: Principles and Methods in Biotechnology;* Wiley: Chichester, UK, 1996.

Kissinger, P.T. J. Pharm. Biomed. Anal. **1996**, *14*, 871–880.

Krull, I.; Swartz, M. LC-GC **1997**, *15*, 534–536, 538, 540.

LaCourse, W.R.; Dasenbrock, C.O. Analytical Reviews **1998**, *70*(12), 37R–52R, 251R–278R; 591R–644R.

Lloyd, D.K. High Perform. Liq. Chromatogra **1996**, *114–42.*

Lough, W.J., Wainer, I.W., Eds. *High Performance Liquid Chromatography: Fundamental Principles and Practice;* Blackie: Glasgow, UK, 1996.

Mant, C.T., Hodges, R.S., Eds. *High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation;* CRC Press: Boca Raton, FL, 1991.

Neue, U.D. *HPLC Columns: Theory and Practice*; Wiley-VCH: New York, 1997.

Noctor, T. High Perform. Liq. Chromatogr. **1996**, *97–113.*

Raghavan, R.; Joseph, J.C. *Encyclopedia of Pharmaceutical Technology*; Swarbrick, J., Boylan, J., Eds.; Marcel Dekker, Inc.: New York, 1993; 7, 249–298.

Riley, C.M., Rosanske, T.W., Eds. Progress in Pharmaceutical and Biomedical Analysis. *Development and Validation of Analytical Methods*; Pergamon Press/Elsevier Science: New York, 1996; 3.

Rothman, L.D. Anal. Chem. **1996**, *68*, 587–598.

Snyder, L.R., Kirkland, J.J., Galajch, J.L., Eds. *Practical HPLC Method Development,* 2nd Ed.; Wiley: New York, 1997.

Swadesh, J., Ed. *HPLC: Practical and Industrial Applications*; CRC Press: Boca Raton, FL, 1997.

Swartz, M.E.; Krull, I.D. *Analytical Method Development and Validation*; Marcel Dekker, Inc.: New York, 1997.

U.S. Pharmacopeia 24/National Fomulary 19, United States Pharmacopeial Convention. Rockville, MD, 2000.

U.S. Pharmacopeia XXIII/National Fomulary XVIII, United States Pharmacopeial Convention. Rockville, MD, 1995.

U.S. Pharmacopeia XXII/National Fomulary XVII, United States Pharmacopeial Convention. Rockville, MD, 1990.

Weston, A.; Brown, P.R. *HPLC and CE: Principle and Practice*; Academic Press: San Diego, CA, 1997.